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A Subcomplex of the Proteasome Regulatory Particle Required for Ubiquitin-Conjugate Degradation and Related to the COP9-Signalosome and eIF3

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Summary

The proteasome consists of a 20S proteolytic core particle (CP) and a 19S regulatory particle (RP), which selects ubiquitinated substrates for translocation into the CP. An eight-subunit subcomplex of the RP, the lid, can be dissociated from proteasomes prepared from a deletion mutant for Rpn10, an RP subunit. A second subcomplex, the base, contains all six proteasomal ATPases and links the RP to the CP. The base is sufficient to activate the CP for degradation of peptides or a nonubiquitinated protein, whereas the lid is required for ubiquitin-dependent degradation. By electron microscopy, the base and the lid correspond to the proximal and distal masses of the RP, respectively. The lid subunits share sequence motifs with components of the COP9/signalosome complex and eIF3, suggesting that these functionally diverse particles have a common evolutionary ancestry.

Introduction

The proteasome appears to be the major protease of the nuclear and cytoplasmic compartments of the eukaryotic cell (Coux et al., 1996; Larsen and Finley, 1997; Baumeister et al., 1998; Rechsteiner, 1998). Substrates are targeted to the proteasome primarily by conjugation to ubiquitin. The ubiquitin-proteasome system plays important regulatory roles in a variety of cellular process, including cell cycle control, antigen presentation, signal transduction, DNA repair, transcriptional silencing, neuronal pathfinding, and long-term facilitation of withdrawal reflexes in *Aplysia* (Hershko and Ciechanover, 1998).

The proteasome is the most complex proteolytic assembly known. It is a labile structure that can dissociate into a 20S core particle (CP) and a 19S regulatory particle (RP; the RP is also referred to as PA700 in mammals and the μ particle in *D. melanogaster*). In mammalian cells, the CP can also associate with complexes other than the RP, such as PA28/Reg (Dubiel et al., 1992; Ma et al., 1992; Gray et al., 1994; Realini et al., 1997) and PI31 (Chu-Ping et al., 1992). The CP of *S. cerevisiae* contains 14 subunits (Heinemeyer et al., 1994), and the RP at least 18 (Fujimuro et al., 1998; Glickman et al., 1998). The proteolytic active sites of the proteasome are found in the CP, sequestered within the lumen of this cylindrical complex (Groll et al., 1997). Proteins apparently enter the CP through channels located at each cylinder end. The free CP, however, does not degrade ubiquitin-protein conjugates.

The CP is activated for proteolysis by binding of the RP to form the proteasome holoenzyme. The RP binds the outer port of the CP channel, implying that the RP initiates substrate translocation into the CP (Larsen and Finley, 1997; Baumeister et al., 1998). In *T. acidophilum*, the channel leading into the CP is only 13 Å in diameter (Löwe et al., 1995), suggesting that translocation may require prior unfolding of the substrate, perhaps by the RP itself. These data, as well as studies of the binding of free multiubiquitin chains to the proteasome (Pickart, 1997), indicate that the selection of ubiquitinated proteins for degradation is mediated by the RP. Whereas the free CP is competent to hydrolyze small peptides, its specific activity on these substrates is less than that of the proteasome holoenzyme (Hoffman and Rechsteiner, 1994; Ma et al., 1994; Glickman et al., 1998). This observation probably reflects that, in the free form of the CP of *S. cerevisiae*, its channel exists predominantly in a closed state (Groll et al., 1997). Thus, an additional role of the RP may be to mediate gating of the substrate channel of the CP.

A striking feature of the RP is that six of its subunits are ATPases (Rpt1-Rpt6). This suggests that the RP may function analogously to ATPase ring complexes such as GroEL, which have been implicated in the facilitation of protein folding (Fenton and Horwich, 1997; Bukau and Horwich, 1998). The RP is an unusually complex ATPase assembly both because of the diversity of its ATPases and because in the RP the ATPases are incorporated into a larger particle with at least 12 other non-ATPase subunits (Rpn1–12). Thus, a better understanding of the RP would provide a new perspective on our understanding of multisubunit ATPase complexes.

In this work, we define two discrete subcomplexes derived from the RP in vitro. We show that the base of the RP, which binds to the CP, contains all six of the ATPases, as well as the non-ATPase subunits Rpn1/Nas1, Rpn2/Sen3, and Rpn10/Mcb1. The remainder of the RP subunits form a discrete complex, the lid, which is distal to the base. The base complex alone can activate the CP for degradation of peptides and a nonubiquitinated protein, suggesting that it is competent to gate the channel of the CP. The base and the lid domains are, however, jointly required for degradation of ubiquitin-protein conjugates. Remarkably, the lid complex exhibits structural similarities to eIF3, a mediator of translational initiation (Hershey et al., 1996; Asano et al., 1997a,

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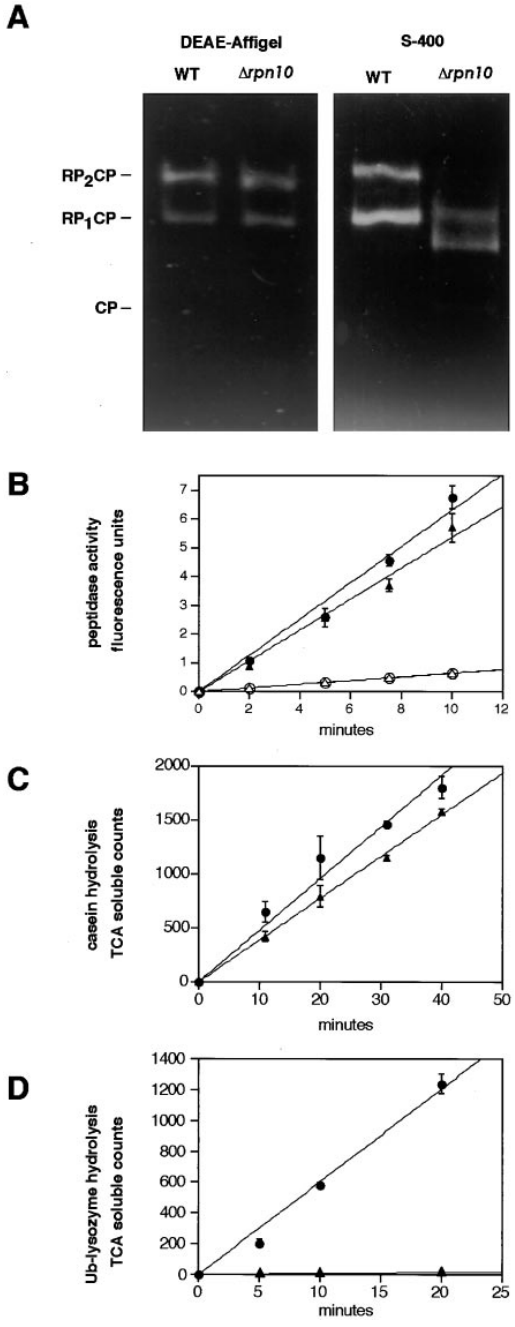

Figure 1. Comparison of Proteasomes Purified from Wild-Type and Δ*rpn10* Cells

(A) Wild-type and Δ*rpn10* lysates were fractionated on columns containing DEAE-Affigel blue, Resource Q, and S400 resin (see Experimental Procedures). Fractions from the DEAE-Affigel and S400 columns containing the peak of peptidase activity were visualized by nondenaturing PAGE and fluorogenic peptide overlay. The observed species contained either one regulatory particle (RP_1CP) or two (RP_2CP). A faint species corresponds to free core particle (CP). Purified Δ*rpn10* fractions from the S400 column contain two faster-migrating species with peptidase activity.

(B) Purified proteasomes (30 μg) from both strains were tested for the ability to hydrolyze the fluorogenic peptide suc-LLVY-AMC. Peptidase activity is given in arbitrary fluorescence units. Closed circles, wild-type proteasome holoenzyme assayed in the presence of ATP; open circles, wild-type proteasomes preincubated (and assayed) in the absence of ATP to dissociate the RP from the CP; closed triangles, Δ*rpn10* in the presence of ATP; open triangles, dissociated Δ*rpn10* proteasomes assayed in the absence of ATP.

(C and D) Purified proteasomes from both strains were tested for the ability to hydrolyze ^{14}C-labeled casein or multiubiquitinated ^{125}I-labeled lysozyme in the presence of ATP. Degradation is measured as the production of TCA-soluble CPM at a given time point. Background radioactivity was subtracted from all readings.

1997b), and the COP9/signalosome complex required for signal transduction during light-induced germination in *A. thaliana* (Wei et al., 1994; Chamovitz and Deng, 1998; Hofmann and Bucher, 1998; Seeger et al., 1998; Wei et al., 1998). The assignment of distinct functions of the RP to stable subcomplexes provides a powerful new approach to the mechanistic analysis of the proteasome.

Results

Purification of the Proteasome from Δ*rpn10* Mutants

We have previously shown that Rpn10/Mcb1 is a nonessential subunit of the proteasome (van Nocker et al., 1996). To investigate the function of Rpn10, proteasomes were partially purified from a strain in which the corresponding gene had been deleted (Δ*rpn10*). Proteasomes were resolved by nondenaturing PAGE and visualized using a fluorogenic peptide overlay assay. Both wild-type and Δ*rpn10* samples contained a mixture of doubly capped (RP_2CP) and singly capped (RP_1CP) proteasomes (Figure 1A, left panel). Upon further purification, however, the two forms of proteasome in the Δ*rpn10* preparation migrated faster than wild-type proteasomes (Figure 1A, right panel).

When the proteasome holoenzyme is formed by the association of the RP and the CP, an important consequence is that the peptidase activity of the CP is stimulated from its basal state (Ma et al., 1994; Glickman et al., 1998). The fast-migrating Δ*rpn10* proteasomes had approximately wild-type levels of peptidase activity (Figure 1B), indicating that the ability of the RP to stimulate peptidase activity was not significantly affected in the purified mutant proteasomes. A slight reduction of activity in the mutant sample was apparent, however. As a control, proteasomes were preincubated in the absence of ATP to release their core particles. The peptidase activities of the dissociated wild-type and Δ*rpn10* samples were comparable, as expected, indicating that basal peptidase activity is not altered by the mutation.

Purified Δ*rpn10* proteasomes were also competent for the degradation of a nonubiquitinated protein substrate, casein (Figure 1C). In contrast, when ubiquitin-protein conjugates were used as substrates, no degradation could be detected in the mutant sample (Figure 1D). Δ*rpn10* proteasomes at an earlier stage of purification (corresponding to Figure 1A, left panel) are capable of degrading ubiquitin-protein conjugates with an activity close to that of wild-type (data not shown). Moreover, Δ*rpn10* mutants are competent to degrade several ubiquitin-protein conjugates in vivo (van Nocker et al., 1996; Fu et al., 1998). Thus, the ability of Δ*rpn10* proteasomes to degrade ubiquitin-protein conjugates was apparently

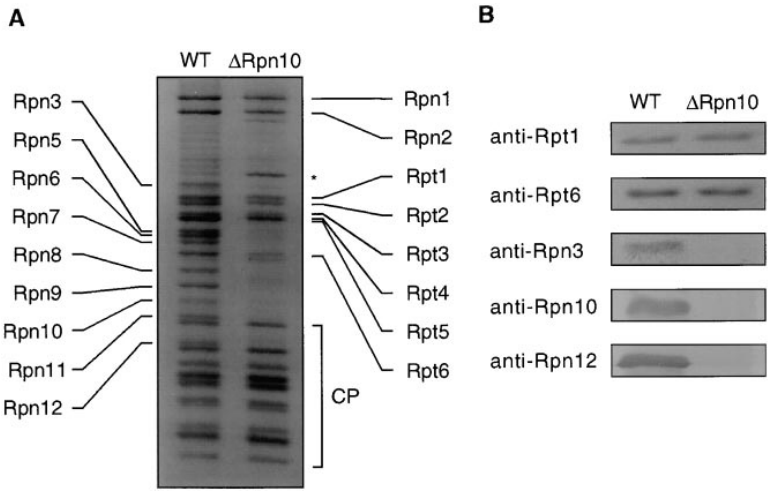

Figure 2. Subunit Composition of the Δ*rpn10* Proteasome

(A) Proteins from purified wild-type and Δ*rpn10* proteasomes were resolved on a 10%–20% polyacrylamide gradient gel. Protein bands were stained with Coomassie blue. The wild-type RP contains 17 protein bands in the 120 kDa to 30 kDa region; band assignments are based on direct amino acid sequence analysis as described (Glickman et al., 1998). The RP from Δ*rpn10* contains eight subunits. Subunits listed at left are present in the RP of wild-type but absent in the RP from Δ*rpn10*. In the Δ*rpn10* sample, the relative levels of CP subunits are slightly higher, apparently because Δ*rpn10* proteasomes are smaller than those of wild-type and thus not as well resolved from the CP complex during the final, gel filtration step of purification. Similarly, minor contaminating species vary between the preparations presumably because the two complexes have different molecular masses and elute in different S400 fractions. The asterisk indicates a protein that is apparently not a subunit of the proteasome.

(B) Immunoblots of purified wild-type and Δ*rpn10* proteasomes resolved by SDS-PAGE and probed with the indicated antibodies.

lost in parallel with the electrophoretic mobility shift during the course of purification. Furthermore, these data show that activation of the core particle for peptide hydrolysis can be uncoupled from ubiquitin-conjugate degradation.

The Base of the Regulatory Particle

To test for possible compositional differences between mutant and wild-type proteasomes, the two purified samples were analyzed by SDS-polyacrylamide gradient gel electrophoresis (Figure 2). We have previously identified 17 subunits of the *S. cerevisiae* RP, which range from 30 to 120 kDa in mass (Glickman et al., 1998). An additional subunit, Rpn4/Son1 (Fujimuro et al., 1998), does not appear to be present in the purified form of the complex. Remarkably, eight subunits were missing from the RP of the mutant strain (in addition to Rpn10 itself): Rpn3, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn11, and Rpn12. This observation accounts for the altered electrophoretic mobility of purified Δ*rpn10* proteasomes. The remaining subunits (Rpn1, Rpn2, and the ATPase family Rpt1 to Rpt6) were present at levels comparable to one another. Based on the results of Figure 1, these are the RP subunits that attach to the CP and activate it for peptide and casein hydrolysis. However, degradation of ubiquitin-protein conjugates requires the intact RP.

When purified proteasomes from wild-type yeast were examined by electron microscopy (Figure 3), they were found to be closely similar to those previously studied from metazoans (Peters et al., 1993). Two forms of the proteasome were observed, containing either one copy of the RP per CP (RP_1CP; Figure 3A), or two copies of the RP per CP (RP_2CP; Figure 3B). In a two-dimensional projection derived from averaged images, the wild-type regulatory particle is highly asymmetric and resembles an open wedge, the proximal arm of which is bound to the core particle. The mutant proteasomes were also found in two predominant forms, consistent with the nondenaturing gel analysis of Figure 1A. These forms are analogous to the wild-type RP_1CP and RP_2CP complexes, except that the RP portion of the mutant complexes is smaller and altered in appearance (Figure 3C and 3D). The mutant RP appeared to be largely symmetric and hemispherical in form.

It is surprising that the Δ*rpn10* proteasomes, despite their markedly altered structure and composition, are comparable to wild-type in several activity assays. In comparison to wild-type, the key structural feature of Δ*rpn10* proteasomes is that they are missing the distal arm of the RP. This implies that the eight dissociable subunits may constitute the distal mass of the RP. The proximal mass, including Rpn1, Rpn2, and Rpt1-Rpt6, will be referred to the *base* of the regulatory particle.

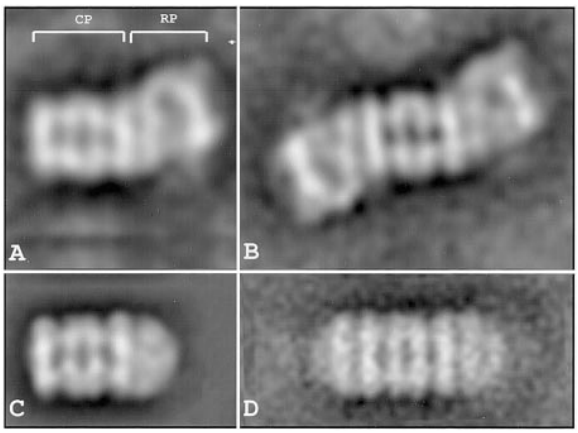

Figure 3. Electron Micrographs of Proteasomes Purified from Wild-Type and Δ*rpn10* Cells

Wild-type and Δ*rpn10* mutant proteasomes were adsorbed onto a carbon film and stained with 2% uranylacetate. Each image represents an averaged data set.

(A) Wild-type RP_1CP complexes (asymmetric form; length, 29 nm).

(B) Wild-type RP_2CP complexes (symmetric form; 41 nm).

(C) Δ*rpn10* mutant complexes (asymmetric form; 21 nm).

(D) Δ*rpn10* mutant complexes (symmetric form; 27 nm).

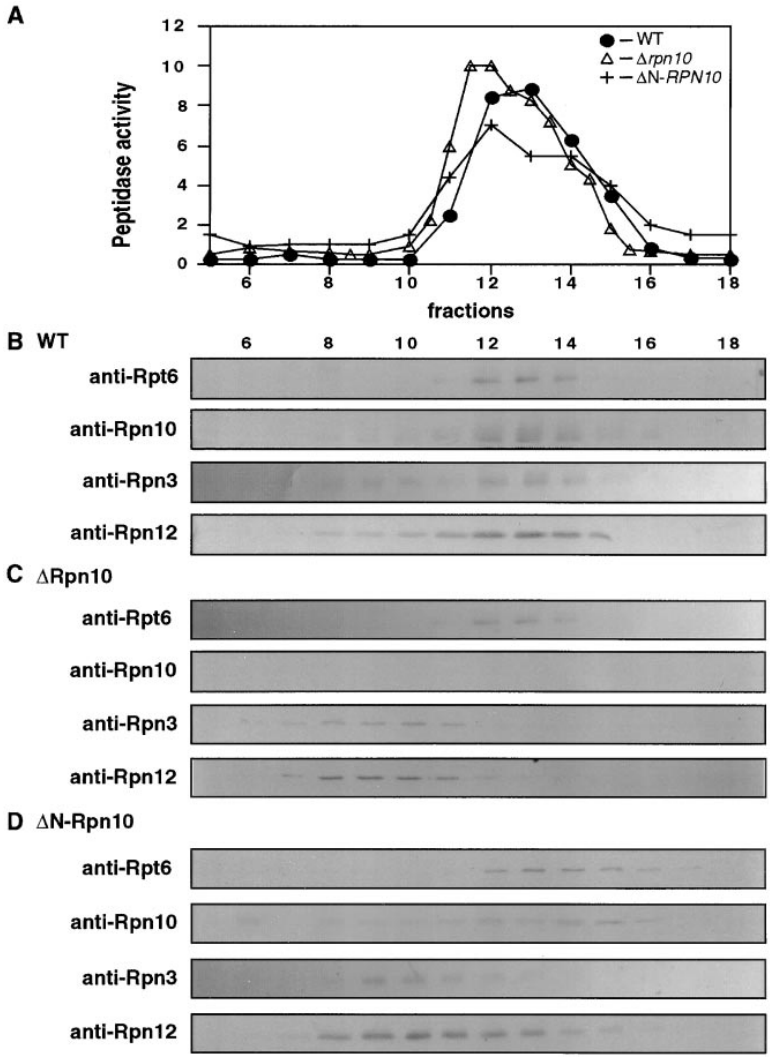

Figure 4. Anion Exchange Chromatography of Proteasomes from Wild-Type, Δ*rpn10*, and Δ*N-rpn10* Strains

Lysates were fractionated on a column containing DEAE-Affigel blue. The peak of peptidase activity was then resolved on a Resource Q column. In the Δ*N-rpn10* strain, the N-terminal 61 residues are deleted from Rpn10.

(A) Fractions from the Resource Q column were tested for the ability to hydrolyze the fluorogenic peptide suc-LLVY-AMC.

(B) Fractions from the Resource Q column were resolved by SDS-PAGE and probed with the indicated antibodies.

The mass density of the base in Δ*rpn10* proteasomes predominantly overlaps with the proximal arm of the wild-type RP (Figure 3).

Structural Role of Rpn10

Although the C termini of Rpn10 and its homologs in other eukaryotes, such as S5a and Mbp1, have been found to bind ubiquitin chains in vitro (Deveraux et al., 1994; Haracska and Udvardy, 1997; Fu et al., 1998; Young et al., 1998), this region of Rpn10 is dispensable in vivo (Fu et al., 1998). In contrast, deletion of the N-terminal 61 codons of *RPN10* produces phenotypes comparable to those of a complete deletion (van Nocker et al., 1996; Fu et al., 1998). It is unknown why the Rpn10 N terminus is required for specific aspects of proteasome function. To determine the localization of Rpn10 in the proteasome and elucidate its role in maintaining the structure of the complex, extracts from wild-type, Δ*rpn10*, and Δ*N-rpn10* (a deletion removing the N-terminal 61 amino acids) strains were studied.

The extracts were fractionated on DEAE-Affigel blue, and fractions containing the peak of activity against Suc-LLVY-AMC were further purified by chromatography on MonoQ anion exchange resin. The three samples exhibited similar peptidase activity profiles (Figure 4A). In wild-type extracts, the peak of peptidase activity, corresponding to the intact proteasome, was coincident with the peaks of Rpt6, Rpn3, Rpn10, and Rpn12, as expected. However, in Δ*rpn10* extracts, Rpn3 and Rpn12 eluted at 230 mM NaCl, preceding the peak of peptidase activity, which, similarly to the wild-type proteasome, elutes at approximately 330 mM NaCl (Figure 4C). Rpn3 and Rpn12 also fractionated separately from the proteasome in the Δ*N-rpn10* sample (Figure 4D). Notably, the N-terminally truncated Rpn10 cofractionated with proteasome peptidase activity following dissociation of Rpn3 and Rpn12, suggesting that Rpn10 is a component of the base (Figure 4D). The base-core particle complex can also be generated from wild-type proteasomes when harsher methods are used for the dissociation step, such as higher concentrations of salt. Analysis of such complexes by nondenaturing gel electrophoresis, followed by a second dimension of SDS-PAGE and immunoblotting, has shown that Rpn10 is present in the wild-type base (data not shown). In summary, Rpn10 associates with the base of the RP, and its N terminus

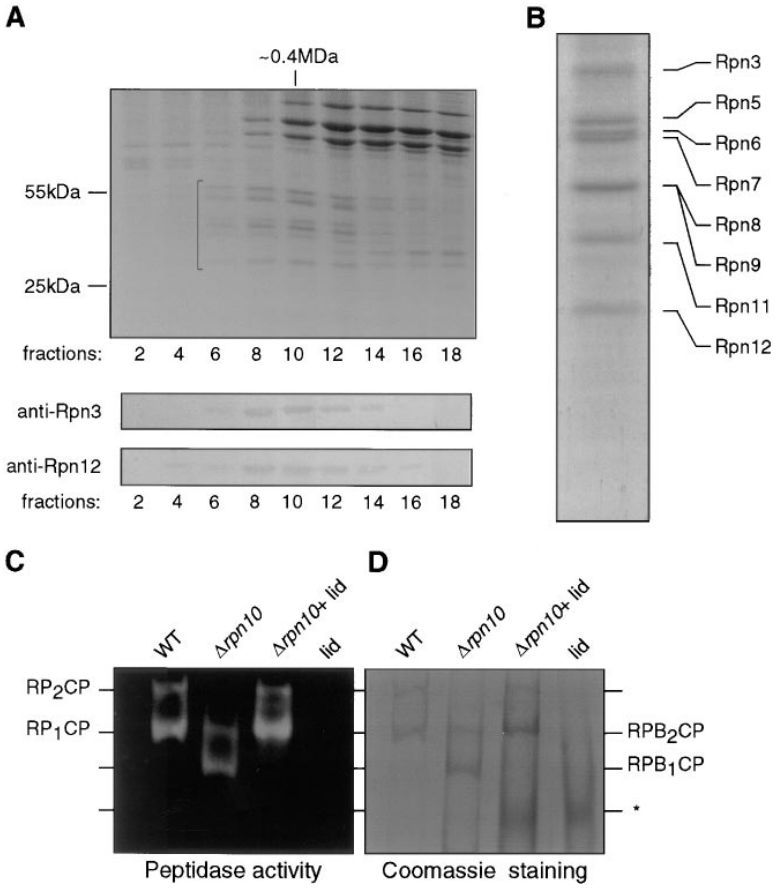

Figure 5. Purification of the Lid Complex

Δ*rpn10* lysate was sequentially fractionated on columns containing DEAE-Affigel blue, Resource Q, S300, and heparin-Sepharose. S300 fractions 8–12, eluting at ~400 kDa, were further purified by heparin-Sepharose chromatography, using a 0–500 mM gradient of NaCl in buffer A without ATP.

(A) Above, SDS-PAGE analysis of S300 column fractionation. Elution volume of a protein marker with a molecular mass of ~400 kDa is indicated by an arrow. Below, immunoblot analysis of the same fractions, using the antibodies indicated. The bracket indicates components of the lid complex.

(B) Gradient SDS-PAGE analysis of the peak of the heparin-Sepharose column fraction eluting at ~400 mM NaCl. Proteins were stained with Coomassie blue. Band assignments for Rpn3 and Rpn12 were reached through immunoblot analysis, (A), and for the other subunits by direct amino acid sequence analysis (see Experimental Procedures). Contaminating protein of high molecular mass remains at this stage of the purification (not shown).

(C) Reconstitution of the RP. Purified lid complexes, as shown in (B), were incubated with purified proteasomes from Δ*rpn10* at 4-fold molar excess in buffer A for 30 min at 30°C. The samples were then analyzed by nondenaturing PAGE and compared to wild-type and Δ*rpn10* proteasomes, using the fluorogenic peptide overlay assay. RPB, base of the RP; Asterisk, unassigned band that is likely to correspond to the lid.

(D) Coomassie blue stain of the gel shown in (C).

plays a role in maintaining the structural integrity of the RP complex.

The Lid, a Distal Subcomplex of the RP

The coelution of Rpn3 and Rpn12 from the MonoQ column (Figure 4C) suggested that the dissociated subunits of the regulatory particle may form a discrete complex. To test this possibility, the MonoQ fractions containing Rpn3 and Rpn12 were subjected to gel filtration chromatography and the molecular masses of complexes containing Rpn3 and Rpn12 were determined. Rpn3, a 60 kDa protein, and Rpn12, a 32 kDa protein, coeluted at ~400 kDa (Figure 5A), suggesting they are part of a distinct complex containing additional subunits. A complex containing all eight of the released subunits would be predicted to have a molecular mass of approximately 360 kDa.

The ~400 kDa complex was further purified using a heparin-Sepharose column. The complex was eluted with a gradient of NaCl, and fractions were resolved by SDS-polyacrylamide gradient gel electrophoresis. The set of cofractionating proteins was found to contain eight distinct species in approximately equimolar amounts (Figure 5B). To verify that each putative component of this complex is derived from the proteasome, the eight protein bands resolved in Figure 5B were individually identified. Rpn3 and Rpn12 were identified by immunoblotting (Figure 5A and data not shown), while Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, and Rpn11 were identified through Edman degradation (see Experimental Procedures). The results confirmed that each of the dissociable subunits of the RP is recovered in the isolated complex. These experiments, together with the electron microscopy data of Figure 3, indicate that the ~400 kDa particle corresponds to the distal mass of the regulatory particle. We will refer to this stable subcomplex as the *lid* of the proteasome.

The finding that all eight of the dissociable subunits are released in the form of a single particle suggested the feasibility of reconstituting proteasomes by incubating purified Δ*rpn10* proteasomes in the presence of lid particles. Because the release of the lid occurred when the salt concentration was raised, low salt conditions were chosen for reconstitution. Reconstitution was assayed by nondenaturing gel electrophoresis. As shown in Figure 5C and 5D, the RP_2CP and RP_1CP forms of the proteasome were efficiently regenerated in the mixed samples and comigrated with wild-type proteasome holoenzyme. Therefore, the lid can reassociate with Δ*rpn10* proteasomes, even in the absence of Rpn10.

Discussion

Two Domains within the Regulatory Particle

The functional dissection of large protein complexes such as the RP has often been accomplished through

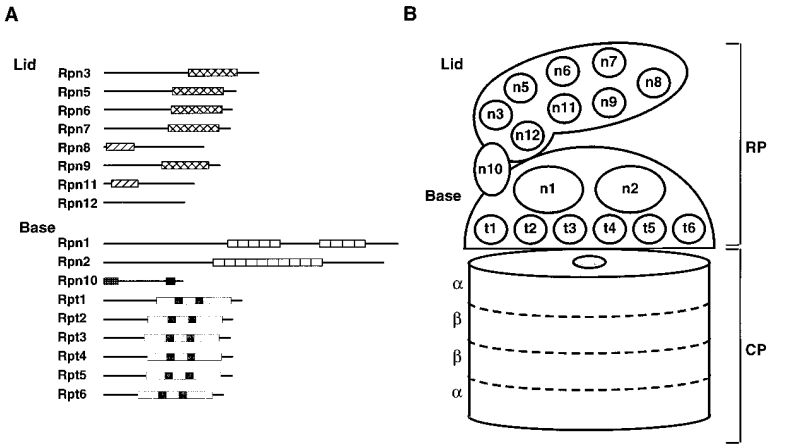

Figure 6. Subunit Organization of the Proteasome Regulatory Particle

(A) Summary of identified domains in regulatory particle subunits. Hatched box, PINT/PCI domain (Aravind and Ponting, 1998; Hofmann and Bucher, 1998); striped box, MPN domain (Hofmann and Bucher, 1998); open boxes, repeat motif with similarities to the LRR motif (Lupas et al., 1997). Rpn1 contains nine such repeats, whereas Rpn2 contains ten; checkered box, N-terminal conserved domain I of Rpn10, which is contained within the N-terminal deletion (residues 1–61) used in this work (Fu et al., 1998). Domain I is contained within a larger sequence related to the von Willebrand factor A (vWF-A) domain (K. Hofmann, personal communication; see also Celikel et al., 1998). In Rpn10, this motif spans residues 1–195. Black box, the conserved domain III of Rpn10, containing the in vitro ubiquitin chain–binding site (Fu et al., 1998); stippled boxes represent AAA cassettes (an ATPase domain [Beyer, 1997]). The darker regions within the cassettes correspond to the highly conserved Walker A and B motifs (Walker et al., 1982; Beyer, 1997). The sequence motifs that are most conserved among different RP proteins are the AAA cassettes. The N-terminal portion of the PINT/PCI motif of Rpn9 is relatively divergent (K. Hofmann, personal communication). All domains are drawn to scale.

(B) A model for the regulatory particle. The proteasome is composed of two major particles: the CP and the RP. The RP contains the ~600 kDa base and ~360 kDa lid subcomplexes. Within the base are the six ATPases, or Rpt proteins; the two largest subunits, Rpn1 and Rpn2; and Rpn10. The remaining eight Rpn subunits comprise the lid. Because the association of the lid and the base is relatively unstable in the absence of Rpn10, this subunit is depicted at the interface of the two subcomplexes. The detailed arrangement of subunits within the lid and base complexes is arbitrary. The CP consists of two types of heptameric rings of subunits (α and β).

the definition of discrete subcomplexes. By characterizing the biochemical activities of individual subcomplexes, the steps in the reaction pathway can be resolved from one another, studied in isolation, and mapped to specific subunits. It is well established that the proteasome can be dissociated into two particles, the CP and the RP. We now show that the RP can be further dissected into two subcomplexes, which we refer to as the lid and the base. As shown schematically in Figure 6, the lid contains Rpn3, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn11, and Rpn12, and the base contains Rpn1, Rpn2, Rpn10, and all six ATPases, Rpt1–6. These data provide general insights into the subunit organization of the regulatory particle.

At the current 20 Å level of resolution, electron micrographs of the proteasome indicate that a portion of the RP is bound to the cylindrical ends of the core particle, while an additional mass is situated distally, beyond a cavity in the RP (Figure 3). We suggest that these two masses within the RP correspond to the base and the lid, respectively. In support of this model, the base particle binds the CP and the mass density of the base overlaps extensively with that of the proximal mass density of the intact wild-type RP. Moreover, when the lid is removed from the RP, the distal mass density is no longer visualized.

The Lid Belongs to a Family of Multisubunit Particles

Two structural motifs, the PINT/PCI and MPN domains, appear to be present exclusively in multisubunit complexes such as the proteasome, eIF3, and the COP9/signalosome particle (Aravind and Ponting, 1998; Hofmann and Bucher, 1998; see Figure 6 and Table 1). These motifs have been recognized only recently, and it is not yet known whether they are associated with a specific biochemical activity. The PINT/PCI domain is up to 200 residues in length and is predicted to form a set of α helices linked by short loops (K. Hofmann, personal communication). This domain has been found in the C termini of Rpn3, Rpn5, Rpn6, Rpn7, and Rpn9 (Aravind and Ponting, 1998; Hofmann and Bucher, 1998). The MPN domain, found at the N termini of Rpn8 and Rpn11, spans approximately 140 residues and is predicted to assume an α/β structure (Hofmann and Bucher, 1998).

Remarkably, all of the proteasome subunits that possess these motifs are found in the lid rather than the base of the regulatory particle or the core particle. Furthermore, all but one of the subunits of the lid contain either an MPN or PINT/PCI motif. Similarly, all eight subunits of the COP9/signalosome complex contain MPN or PINT/PCI motifs (Wei et al., 1998). The distribution of the motifs is similar between the two complexes:

Table 1. A Family of Multisubunit Complexes with Common Structural Motifs

Complex	Function	kDa	Total Subunits	Subunits with MPN motifs	Subunits with PINT/PCI Motifs	References
RP lid	protein degradation	400	8	2	5	this work
COP9/Signalosome	signal transduction	450	8	2	6	(Seeger et al., 1998; Wei et al., 1998)
eIF3	initiation of translation	600	10	2	3	(Asano et al., 1997a, 1997b)

MPN motifs are found in two subunits of each complex, while PINT/PCI motifs are found within six subunits of the COP9/signalosome complex and in five subunits of the lid. eIF3 also contains two subunits with MPN motifs, but only three with PINT/PCI motifs (Hofmann and Bucher, 1998). Finally, both the COP9/signalosome complex and eIF3 contain one subunit with specific similarity to Rpn8 and one with specific similarity to Rpn11. Among the lid subunits, Rpn11 is not only the most closely related to any component of the COP9 complex, but is also substantially more conserved in evolution than other subunits of the lid (Glickman et al., 1998).

The identification of the lid as a distinct subcomplex of the regulatory particle provides an important basis for the interpretation of these structural relationships. Indeed, identification of the lid helps to define a new family of multisubunit assemblies, each of which is broadly distributed among eukaryotes. Seeger et al. (1998) have proposed that the homologies between proteasome and COP9/signalosome complex subunits reflect common substrate-binding sites, while Hofmann and Bucher (1998) have suggested that the existence of PINT/PCI and MPN domains in eIF3 and the COP9/signalosome complex may indicate that these complexes, like the RP, may interact with the proteasome core particle. We propose that the relationship among these particles reflects a common evolutionary ancestry and that a key step in the evolution of the modern proteasome may have been the development of a binding interaction between a precursor of the lid and a precursor of the base. Simple homologs of the proteasome have been described in prokaryotes, in which the RP consists only of six identical ATPases (Wolf et al., 1998; P. Zwickl and A. Goldberg, personal communication). The composition of the base particle that we have identified (Figure 6) is not markedly different from this possible evolutionary precursor. The ATPase subunits have diversified in eukaryotes to form a hetero-oligomer (Glickman et al., 1998) and have also differentiated functionally (Rubin et al., 1998), but have not changed in number. In addition, three new subunits that are presumably not ATPases, Rpn1, Rpn2, and Rpn10, have been added to the base.

A clue to the possible functional relationships among the lid, the COP9/signalosome complex, and eIF3 is that each may interact with a second high–molecular weight factor or protein complex. In the case of the COP9/signalosome complex, the identity of the interacting factor is unknown, but it can be visualized in partially purified preparations (Wei et al., 1994; Chamovitz and Deng, 1998; Seeger et al., 1998). For eIF3, the second complex is the 40S ribosomal subunit; a major function of eIF3 is to promote the binding of initiator tRNA and mRNA to the 40S ribosomal subunit. eIF3 also interacts with eIF4B, eIF4F, and eIF4G (Hershey et al., 1996; Asano et al., 1997a, 1997b). Thus, eIF3 can be viewed as a docking site that escorts a variety of factors to the ribosome. It is plausible that the lid, being the part of the RP that is most exposed to the cytoplasm, also functions as a docking site, with the base playing the critical role of initiating translocation of the substrate in analogy to the role of the 40S ribosomal subunit in the initiation of translation. Given that the lid and the base are both necessary for proteasome activity, each is also a possible target for regulating proteasome function.

Models for Functional Cooperation between the Base and the Lid

An important property of the base is that it is nearly as efficient as the proteasome itself in stimulating the degradation of peptides and the nonubiquitinated protein substrate casein (Figure 1). Consistent with the hypothesis that the base is necessary for activating the CP, peptide hydrolysis by the core particle can be inhibited by mutations in the ATP-binding site of Rpt2, a subunit of the base (Rubin et al., 1998). We suggest that both observations reflect a role of the base in opening of the channel of the core particle. This model is consistent with structural data indicating that the channel is closed in free core particles from yeast (Groll et al., 1997).

Although sufficient to stimulate peptide hydrolysis, the base failed to promote the degradation of a ubiquitinated protein substrate. A simple model accounting for these results is that ubiquitin-protein conjugates might interact with the lid through their ubiquitin moiety and with the base through the substrate component of the conjugate. While ubiquitin chains can be bound in vitro by a component of the base, Rpn10, genetic studies have indicated that *RPN10* does not play a required role in the turnover of several ubiquitin-protein conjugates in vivo, at least in yeast (van Nocker et al., 1996; Fu et al., 1998). That the in vivo effects of the *rpn10* deletion mutation are mild is consistent with the observation that the in vitro lability of Δ*rpn10* proteasomes results from supraphysiological salt conditions.

Two subunits of the base, Rpn1 and Rpn2 (Figure 6), have repeat motifs with some similarity to the leucine-rich repeat (LRR) domain, a common site for protein–protein interaction (Lupas et al., 1997). The six ATPases of the base are also likely to function through protein–protein interaction, by analogy to the simple ATP-dependent proteases of prokaryotes, which directly interact with substrates (Gottesman et al., 1997; Suzuki et al., 1997). Thus, it is plausible that all components of the base may engage in direct interactions with proteolytic substrates. This possibility is consistent with structural data, which suggest that the substrate must translocate through the center of the base to gain access to the core particle (Baumeister et al., 1998). It is clear that further experimentation will be required to refine these simple models for substrate-proteasome interactions. Given that lid and base complexes can be purified in significant amounts and can be used to reconstitute the proteasome, it is now feasible to clarify the functional distinctions between these subcomplexes of the regulatory particle.

Experimental Procedures

Yeast Strains and Media

Strain SUB62 (*MAT***a** *his3-Δ200 lys2–801 leu2–3, 2–112 trp1–1 ura3–52*) (Finley et al., 1987) was used as a wild-type control throughout. All strains used in this work are *MAT***a** and are strictly isogenic to SUB62. The Δ*rpn10* strain (SV1) corresponds to the Δ*mcb1* strain of van Nocker et al. (1996). The gene name has been changed (Finley et al., 1998). This strain contains the allele Δ*rpn10::LEU2*, originally referred to as Δ*mcb1::LEU2*. The Δ*N-rpn10* strain corresponds to

the strain *NΔ1* of Fu et al. (1998). This is a derivative of SV1 in which a *TRP1*-marked plasmid expresses Rpn10 with a 61–amino acid N-terminal deletion. Yeast cultures were grown in YPD media at 30°C in a 12 liter fermenter. YPD media consisted of 1% yeast extract, 2% Bacto Peptone, and 2% glucose. Synthetic media, used for growing Δ*N-rpn10*, consisted of 0.7% Difco yeast nitrogen base supplemented with amino acids (except for tryptophan), uracil, and 2% glucose.

Purification of the Proteasome and the Lid

Purification of the proteasome by conventional chromatography was described previously (Glickman et al., 1998). Buffer A, consisting of 50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 10% glycerol, 1 mM ATP (Grade 1, Sigma), and 1 mM dithiothreitol, was used in all in vitro experiments unless otherwise noted. The purification procedure for the Δ*rpn10* proteasome was identical to that of the wild-type complex except that the peak of peptidase activity eluted in slightly lower molecular weight fractions in the S400 step.

Purification of the lid complex from Δ*rpn10* cells involved tracking the presence of Rpn3 and Rpn12 by immunoblotting. Yeast cell extracts were fractionated on DEAE-Affigel blue and Resource Q (Pharmacia) as for the proteasome (Glickman et al., 1998). Resource Q fractions containing Rpn3 and Rpn12 were pooled and concentrated using Ultrafree concentrators with a molecular weight cutoff of 10 kDa (Millipore) and were further resolved by gel filtration. For gel filtration, 300 ml of S-300 resin was packed in an XK26 column (Pharmacia). Samples were run isocratically in buffer A without ATP, supplemented with 100 mM NaCl, at a flow rate of 1.5 ml/min. Four-milliliter fractions were collected, and those containing Rpn3 and Rpn12 were pooled and loaded onto a 1 ml heparin-Sepharose column (Pharmacia). The lid was eluted with a gradient of NaCl at approximately 400 mM in the absence of ATP. These samples were desalted and concentrated by ultrafiltration. Lid subunits were resolved by gradient SDS-PAGE as described previously (Glickman et al., 1998).

Identification of Lid Subunits

Rpn3 and Rpn12 were identified by immunoblotting. For the remaining six proteins, the stained bands were excised from the gel, digested with trypsin, and the peptides generated were resolved by HPLC. The sequences of representative peptides were then determined by Edman degradation, confirming the identity of Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn11. The identified peptides were as follows: for Rpn5, IVDLLASR (residues 62–69) and TYEPVLNEDD (317–326); for Rpn6, IMLNLIDDVK (270–279) and IIEPFE(C)VEI (350–359); for Rpn7, LGFFYNDQLY (177–186); for Rpn8, NSDV(W)FLD(H)N (62–71) and LQDVFNLLPN (229–238); for Rpn9, ITNSFYSTNS (167–176); and for Rpn11, AVAVVVDPIQ (136–145) and VVIDAFR (151–157). For residues indicated in brackets, no clear assignment could be made.

Assays of Proteasome Activity

Assays for the hydrolysis of fluorogenic peptides, ubiquitin-lysozyme conjugates, and casein were performed essentially as described (Glickman et al., 1998; Kisselev et al., 1998; Rubin et al., 1998). To dissociate the RP from the CP (Figure 1B), proteasomes stored in the presence of ATP were diluted into a buffer containing 50 mM Tris (pH 7.4), 1 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 500 mM NaCl, and 1 unit/ml of apyrase. After a 15 min preincubation at 30°C, peptidase activity was assayed under the same conditions as for intact proteasomes, except for the presence of 50 mM NaCl and the omission of Mg-ATP.

Protein concentrations were determined using Coomassie blue plus, with bovine serum albumin as a standard (Pierce). Fluorogenic peptide overlay assays were performed as described (Glickman et al., 1998). Purified proteasome polypeptides were resolved by SDS-PAGE (12%) using standard techniques (Laemmli, 1970). Proteins were either stained in the gel using Coomassie blue or transferred to nitrocellulose for immunoblotting (Glickman et al., 1998).

Electron Microscopy

Micrographs of the mutant proteasomes were taken with a Philips CM12 at a (nominal) magnification of 30,000× and, in the case of wild-type preparation with an EM 420, at a (nominal) magnification of 36,000×. Negatives were digitized with a step size of 15.5 μm, corresponding to 0.53 nm and 0.44 nm, respectively, at the specimen level. For image analyses of wild-type samples, approximately 600 individual particles were extracted and aligned using correlation averaging methods; approximately 500 particles were chosen in the mutant case. Aligned data sets were subjected to eigenvector-eigenvalue data analyses to determine structural variations before averaging.

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